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14. ABSTRACT
About 1/3rd of the Persian Gulf War veterans' exhibit Gulf War Illness (GWI) symptoms, particularly depression, and memory deficits. Chronic exposure to organophosphates (OP) is among multiple causes for GWI, yet its pathobiology remains ill understood. The role of calcium (Ca^{2+}) signaling in memory and mood is well established. In an OP- diisopropyl fluorophosphate (DFP) based rat model of GWI, we observed disruptions in neuronal Ca^{2+} levels ($[Ca^{2+}]i$). This study is aimed at identifying mechanisms underlying elevated $[Ca^{2+}]i$ and investigating whether their therapeutic targeting could improve GWI neurological morbidities. Sustained Ca^{2+} elevations in GWI neurons had their origin in Ca^{2+} release from intracellular Ca^{2+} stores, since the application of ryanodine/ IP_3 receptor antagonist dantrolene or levetiracetam produced greater than 50% reduction in their levels. Treatment with levetiracetam significantly improved symptoms of depression and anxiety in GWI rats. Since Ca^{2+} is a major second messenger molecule, such chronic increases in its levels could produce pathological synaptic plasticity that expresses itself as GWI morbidities. Our studies show that treatment with drugs targeted at blocking intracellular Ca^{2+} release could be effective therapies for GWI.

15. SUBJECT TERMS
Gulf War Illness, Organophosphate, diisopropyl fluorophosphate (DFP), neurological morbidities, neuronal injury, Sprague-Dawley rats, Calcium imaging, Fura-2, Calcium-induced Calcium Release,

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1. Introduction

Approximately 1/3rd of returning soldiers deployed during the Persian Gulf War exhibit chronic multi-symptom illnesses also known as the Gulf War Illness (GWI). There are several confounding factors attributed to the development of GWI, and after reviewing all of the available data, the Research Advisory Committee on Gulf War Veterans' Illnesses has strongly implicated exposure to organophosphates (OPs) as one of the leading cause for GWI [1,2]. Diisopropyl fluorophosphate (DFP) is an OP compound that is used in civilian laboratories as a surrogate nerve gas agent [3,4]. We have used this agent to mimic OP exposure during Gulf War deployment and have observed depressive symptoms and cognitive deficits in rats exposed to repeated, low-dose DFP exposure [3,5]. The hippocampus plays a major role in the limbic system, is essential in memory functioning [6] and plays a major role in pathophysiology of depression [7]. Studies have shown hippocampal dysfunction in Gulf War veterans using both imaging and neuropsychological testing [8-10]. OP-based animal models of GWI have also demonstrated hippocampal and stratial neuronal loss, inflammation, and reduced synaptic transmission underlying the expression of anxiety, mood and memory deficits [11-15]. Thus, hippocampus is an important brain area to investigate in GWI. Calcium is a major second messenger and plays a vital role in cellular signaling, in developing neuronal plasticity which affects behavior, and memory [16,17]. Brief elevations in Ca^{2+} levels are critical to cellular communication and long-term potentiation (learning and memory consolidation). However, our research and that of other investigators have demonstrated that sustained Ca^{2+} elevations particularly in the hippocampal region are detrimental to the cell and are implicated in many neurological disorders that shares symptomatology with GWI neurological morbidities. There has been recent evidence that Ca^{2+}-induced Ca^{2+} release (CICR), which principally consists of the inositiol-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR), plays a distinct role in memory processing and disease state [17-20]. But at present, the role of CICR signaling system in the development of depression and cognitive impairments in GWI is unknown and will be investigated in these studies using a rodent model of GWI developed in our laboratory [3].

2. Keywords

Gulf War Illness, Organophosphate, diisopropyl fluorophosphate (DFP), neurological morbidities, Sprague-Dawley rats, Calcium imaging, Fura-2, Calcium-induced Calcium Release, Dantrolene, Levetiracetam, H-89, U-73122

3. Accomplishments:

The following lists the accomplishments from our project during the year-3 (2016-2017).

3.1 What were the major goals of the project?

The major goals of the projects for year 3 were to identify molecular mechanisms underlying elevated calcium levels observed in hippocampal neurons from GWI rats (year-2). In addition, we also investigated whether blocking these mechanisms will provide relief from the symptoms of depression and anxiety and related neurological co-morbidities in our rat model of GWI.

3.2 What was accomplished under these goals?

We measured intracellular calcium levels, studied mechanisms of Ca^{2+} entry, handling of the intracellular calcium-induced calcium release (CICR) mechanisms in GWI rats displaying

anxiety, depression and cognitive deficits. Effects of drugs targeting the CICR components in relieving GWI neurological morbidities were also investigated.

I. DFP exposure

DFP was prepared fresh daily by dissolving in ice-cold phosphate buffered saline just before the exposure. Rats were injected with DFP (0.5 mg/kg, s.c., 1x daily for 5-days). Control rats received DFP vehicle injections for the same period. Animal health including weight measurement were assessed every day during the exposure and for the next seven days following the end of DFP injections.

II. Estimation of hippocampal intra-neuronal Ca^{2+} levels: In these experiments, control and GWI rats at various time-points post-DFP exposures were utilized to estimate intracellular Ca^{2+} levels. Briefly, rats were decapitated, brains removed and hippocampal slices obtained on a vibrotome. Following enzymatic treatment, hippocampus was removed and triturated to generate a neuronal suspension. Calcium levels were measured using microfluorimetry. These steps are described below:

II a. Isolation of Hippocampal CA1 Neurons and Loading with Fura-2

Acute isolation of CA1 hippocampal neurons was performed by established procedures routinely used in our laboratory [4,21]. Animals were anesthetized with isoflurane and decapitated. Brains were rapidly dissected and placed in 4°C oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (aCSF) consisting of (in mM): 201.5 sucrose, 10 glucose, 1.25 NaH2PO4, 26 NaHCO3, 3 KCl, 7 MgCl2, and 0.2 CaCl2). MK-801 (1 μM) was added to all solutions to increase cell viability and was removed 15 min prior to imaging. Hippocampal slices (450 μm) were cut on a vibrating microtome (Leica Microsystems, Wetzlar, Germany) and then equilibrated for 10 min at 34°C in a piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES)-aCSF solution containing (in mM): 120 NaCl, 25 glucose, 20 PIPES, 5 KCl, 7 MgCl2, and 0.1 CaCl2. Slices were then treated with 8 mg/ml protease in PIPES-aCSF for 6 min at 34°C and rinsed. Enzyme treated slices were visualized on a dissecting microscope to excise the CA1 hippocampal layer which was then triturated with a series of Pasteur pipettes of decreasing diameter in cold (4°C) PIPES-aCSF solution containing 1 μM Fura-2 AM (Invitrogen, Carlsbad, CA). The cell suspension was placed in the middle of 2 well glass-bottomed chambers (Nunc, Thermo Scientific). These glass chambers were previously treated overnight with 0.05 mg/ml poly-L-lysine followed by multiple rinses with distilled water and then further treated with Cell-Tak™ (BD-Biosciences, San Jose, CA) biocompatible cellular adhesive (3.5 μg/cm2) for 30-min, rinsed and air-dried. Neuronal suspension placed in the center of adhesive coated dishes when settled firmly adhered to the bottom. This technique simplified further manipulations on the dissociated neurons. Plates were then incubated at 37°C in a 5% CO_2/95% air atmosphere for 45 min. Fura-2 was washed off with PIPES-aCSF and plates were incubated an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2.

II b. Measurement of $[Ca^{2+}]_i$

Fura-2 loaded cells were transferred to a 37°C heated stage (Harvard Apparatus, Hollington, MA) on an Olympus IX-70 inverted microscope coupled to a fluorescence imaging system (Olympus America, Center Valley, PA) and subjected to $[Ca^{2+}]_i$ measurements by procedures

well established in our laboratory [4,21]. All experiments were performed using a 20X, 0.7 N.A. water immersion objective and images were recorded by an ORCA-ER high-speed digital CCD camera (Hammamatsu Photonics K.K., Japan). Fura-2 was excited with a 75 W xenon arc lamp (Olympus America, Center Valley, PA). Ratio images were acquired by alternating excitation wavelengths (340/380 nm) by using a Lambda 10-2 filter wheel (Sutter Instruments Co., Novato, CA) and a Fura filter cube at 510/540 emission with a dichroic at 400 nm. All image acquisition and processing was controlled by a computer connected to the camera and filter wheel using Metafluor Software ver 7.6 (MDS Analytical Technologies, Downington, PA). Image pairs were captured every 5s and the images at each wavelength were averaged over 10 frames. Background fluorescence is obtained by imaging a field lacking Fura-2. Hippocampal CA1 neurons were identified based on their distinct morphology. These neurons displayed pyramidal shaped cell body, long axon and dendrites and have been demonstrated to be devoid of immunoreactivity for specific protein markers for interneurons, including parvalbumin, cholecystokinin, vasoactive intestinal peptide, somatostatin, and neuropeptide Y. The process of enzymatic treatment and mechanical trituration can add minimal stress during acute dissociation of neurons. However, we have shown previously that the neurons isolated using these procedures exhibit electrophysiological properties identical neurons in slices or in cultures, are viable, and not apoptotic or necrotic.

II c. Calcium calibration

We performed Ca^{2+} calibration determinations as described previously [4,21,22] to provide estimates of absolute $[Ca^{2+}]_i$ concentrations from the 340/380 ratio values. A Ca^{2+} calibration curve was constructed using solutions of calibrated Ca^{2+} buffers ranging from 0 Ca^{2+} (Ca^{2+} free) to 39 μM Ca^{2+} (Invitrogen, Carlsbad, CA). Values from the calibration curve were used to convert fluorescent ratios to $[Ca^{2+}]_i$. Final $[Ca^{2+}]$i were calculated from the background corrected 340/380 ratios using the Grynkiewicz equation:

$$[Ca^{2+}]_i = (K_d \times Sf_2 / Sb_2) \times (R - R_{min}) / (R_{max} - R)$$

where R was the 340/380 ratio at any time; Rmax was the maximum measured ratio in saturating Ca^{2+} solution (39 μM free Ca^{2+}); R_{min} was the minimal measured ratio Ca^{2+} free solution; Sf_2 was the absolute value of the corrected 380-nm signal at R_{min}; Sb_2 was the absolute value of the corrected 380-nm signal at R_{max}; the K_d value for Fura 2 was 224 nM.

III. Behavioral screening assays:

Amongst the GWI morbidities, the neurological deficits such as chronic depression, anxiety and memory impairments are predominant ones. To investigate whether treatment with CICR drugs such as dantrolene and levetiracetam would lead to reduction in the expression of GWI psychiatric abnormalities, we conducted a battery of rodent behavioral assays that identify symptoms of depression, anxiety and cognitive deficits following DFP exposures. Testing was carried out in a quiet, dimly lit room between 0800 to 1400 hrs. Depression was assessed using the Forced Swim Test (FST), and the Elevated Plus Maze (EPM). Memory function was assessed using the Novel Object Recognition (NOR). These tests were described recently in our paper on GWI model development [3,5]. Dantrolene (10 mg/kg, i.p.), Levetiracetam (50 mg/kg, i.p.), H-89 (2 mg/kg, i.p.), and U-73122 (30 mg/kg, i.p.) were administered 30-mins before the behavioral assays.

IV. Data analysis

For comparing the distributions of $[Ca^{2+}]_i$ levels a Chi-square test was used. Data were analyzed and graphs plotted using the SigmaPlot 12.5 software (SPSS Inc, Chicago, IL). All the data that passed the normality test was further subjected to t-test. A value of $p<0.05$ was considered significant for all data analyses.

V. Results

V. a *Estimations of hippocampal neuronal $[Ca^{2+}]_i$*

CA1 neurons from GWI rats manifested $[Ca^{2+}]_i$ of 399 ± 26 nM, that were significantly higher than $[Ca^{2+}]_i$ from age-matched control rats (208 ± 16 nM) ($p<0.05$, one-way ANOVA, n= 8 animals). Analysis of the population distributions of $[Ca^{2+}]_i$ revealed only 2% of age-matched control neurons exhibited $[Ca^{2+}]_i$ greater than 500 nM. In contrast, ~50% neurons isolated from GWI rats exhibited $[Ca^{2+}]_i$ between 250-500 nM and ~18% neurons exhibited $[Ca^{2+}]_i$ greater than 500 nM, indicating a significant right-ward population shift towards higher $[Ca^{2+}]_i$ concentration range ($p<0.001$, Chi-square test, n= 161 neurons, Fig. 1A, 1B, and 1C).

V. *b Mechanism for elevated hippocampal neuronal $[Ca^{2+}]_i$ following DFP exposures*

Application of nifedipine (5 μM), DNQX (10 μM), or $GdCl_3$ (100 μM) did not significantly affect $[Ca^{2+}]$i in GWI neurons. In contrast, application of dantrolene (50 μM) or levetiracetam (100 μM) significantly lowered elevated $[Ca^{2+}]$i in GWI neurons (240 ± 11 nM and 250 ± 19 nM respectively, n= 5 animals, $p<0.05$, t-test, Fig. 2A, 2B).

V. c *Effect of CICR inhibitors on GWI neurological morbidities*

Having identified CICR as a source of elevated $[Ca^{2+}]_i$ in GWI neurons, we investigated whether targeted blockade of this mechanism would also provide relief from the symptoms of GWI neuropsychiatric abnormalities. CICR is manned by two receptor subtypes: IP3R and RyR. Dantrolene is a specific RyR antagonist, while levetiracetam is a mixed CICR inhibitor. H-89 is PKA inhibitor that would prevent phosphorylation of RyRs and U-73122 is PLCγ inhibitor, which would lower IP3 production. Interestingly, all these agents produced varying effects on GWI morbidities. Unfortunately, effects of Dantrolene could not be separated from its effects on muscular relaxation. However, levetiracetam produced the most profound effect on the GWI behavior. Given that levetiracetam is also a FDA approved drug, it raises the possibility that it could be made available for GWI veterans following a fast-track FDA review. Below, we have included results from our levetiracetam studies. All the drug studies data will be included again in the Final Report.

Performance on FST

The FST is an effective test in evaluating the presence of a despair-like state in the DFP exposed rats. GWI rats subjected to FST exhibited increased immobility time of 127.12 ± 11.69 s indicative of a despair-like state. In the presence of levetiracetam (50 mg/kg, i.p.) there was a significant reduction in immobility time (81.08 ± 6.97 s) (n= 6, $p< 0.05$, Fig. 3A).

Performance on EPM

DFP exposed rats also displayed symptoms of anxiety when subjected to EPM test. Repeated, low-dose DFP rats (0.5 mg/kg, 5-days) displayed increased anxiety as characterized by

significantly lower performance in the open arm of the EPM (time in open arm: 16.9 ± 2.5% in controls vs 5.28 ± 1.7% in 0.5 DFP exposed rats) indicating the presence of symptoms of anxiety. In the presence of levetiracetam (50 mg/kg, i.p.) a significant ($p<0.05$, n=6 rats) anxiolytic effect was observed in the GWI group as identified by an improvement in time spent in the open-arm of EPM which was not significantly different from control group (time in open arm: 16.9 ± 2.5% in controls vs 14.2 ± 2.7% in GWI rats, Fig. 3B).

Performance on NOR

The NOR test revealed deficits in recognition memory in DFP exposed rats. In the choice phase of NOR, repeated, low-dose DFP rats (0.5 mg/kg, 5-days) spent more time exploring the old object compared to the new object indicating that these rats did not remember the familiar object. These rats exhibited a discrimination ratio of 0.47 ± 0.08, indicative of impaired recognition memory that was significantly lower compared to age matched control rats (0.86 ± 0.05, n= 6, $p< 0.05$). In the presence of levetiracetam (50 mg/kg, i.p.) a significant improvement on NOR performance was observed (discrimination ratio: 0.72 ± 0.06, Fig. 3C).

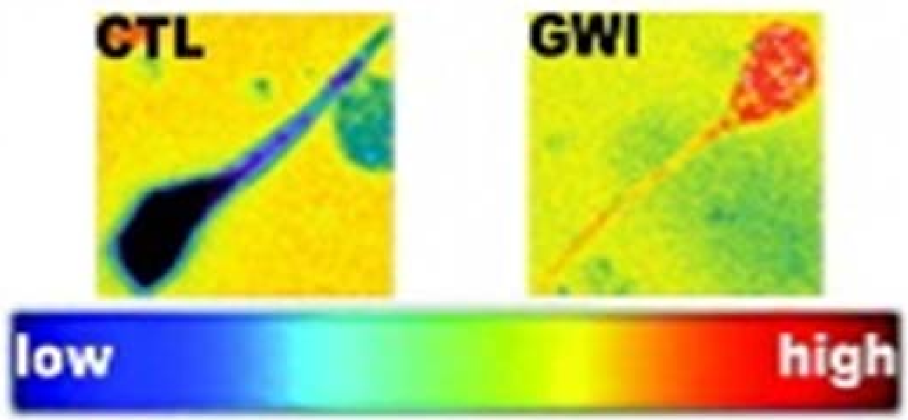

Figure 1A. Psuedocolor images of CA1 hippocampal neurons acutely isolated from GWI rats and age-matched control animals.

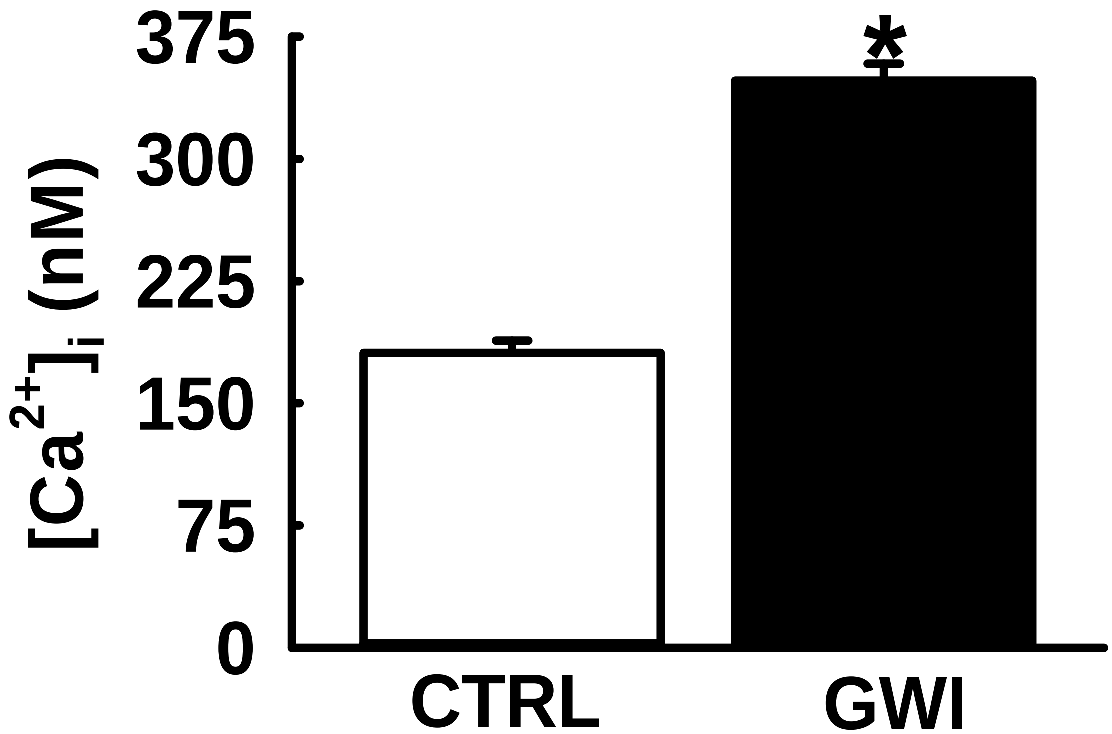

Figure 1B. Elevated $[Ca^{2+}]_i$ in CA1 hippocampal neurons acutely isolated from GWI rats compared to neurons from age-matched control animals. Neuronal Ca^{2+} levels in GWI rats were significantly higher compared to age matched control rats. Data expressed as mean ± SEM, **p*<0.05, t-test, n= 7 rats.

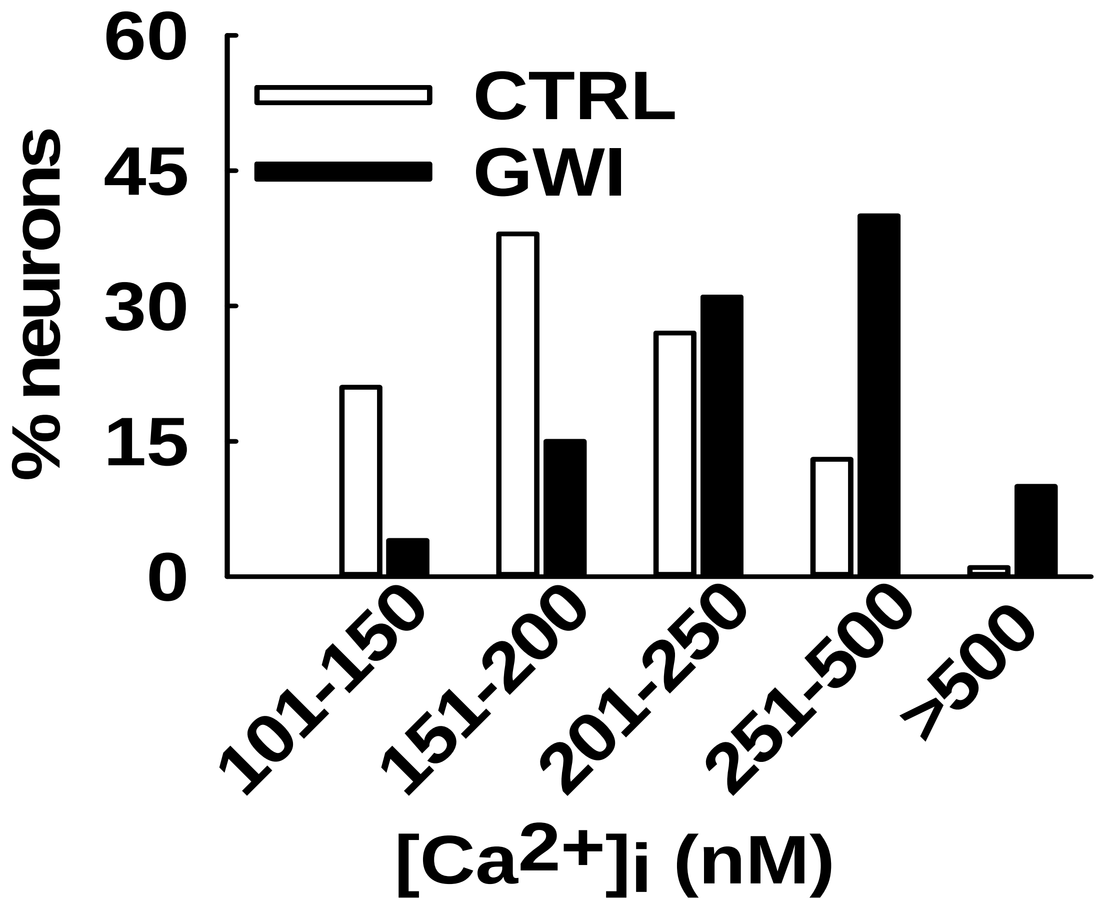

Figure 1C. Distribution of $[Ca^{2+}]_i$ for control and GWI CA1 hippocampal neurons. Control neurons demonstrated a normal distribution for $[Ca^{2+}]_i$ with approximately 95% of neurons exhibiting $[Ca^{2+}]_i$ less than 500 nM and only 5% neurons exhibiting very high $[Ca^{2+}]_i$. In contrast, neurons from GWI rats demonstrated a rightward shift towards higher $[Ca^{2+}]_i$ with approximately 50% neurons exhibiting $[Ca^{2+}]_i$ greater than 500 nM (n= 161 neurons).

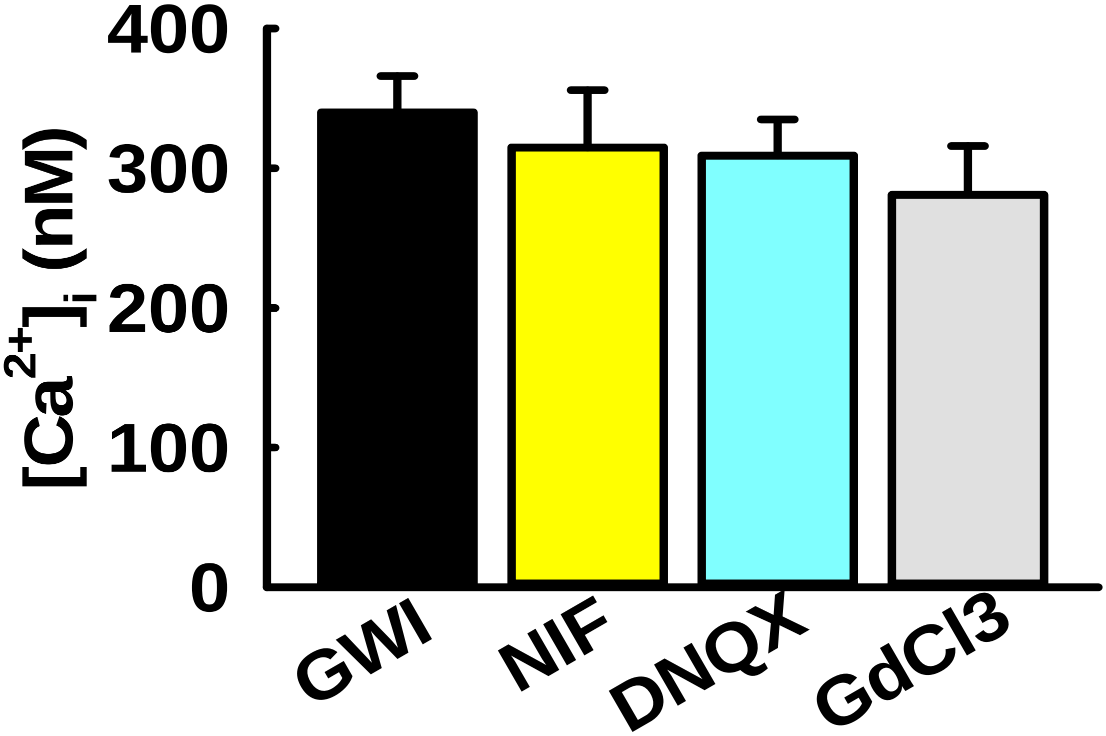

Figure 2A. Mechanism of Ca^{2+} plateau following DFP exposure. CA1 hippocampal $[Ca^{2+}]_i$ from GWI rats (black bar) were not significantly altered in the presence of nifedipine (NIF, 5 μM), or DNQX (10 μM) or GdCl3 (100 μM) (n= 5-6 animals for each treatment). Data represented as mean ± SEM.

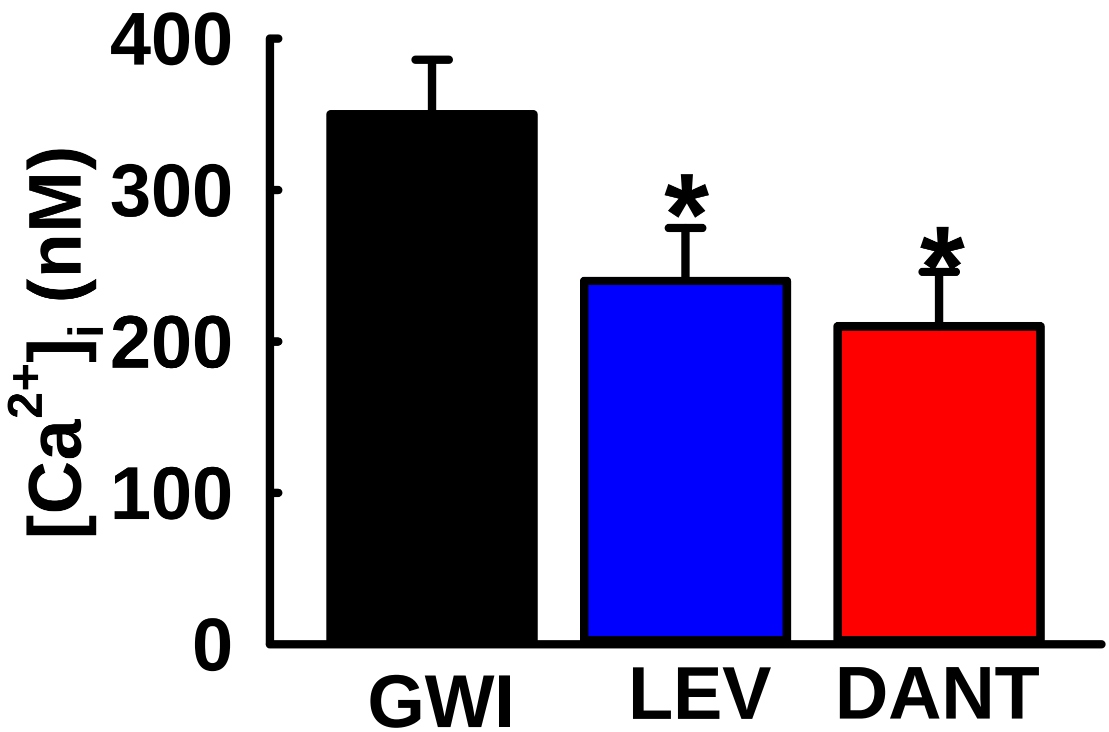

Figure 2B. Mechanism of Ca^{2+} plateau following DFP exposure. CA1 hippocampal $[Ca^{2+}]_i$ from GWI rats (black bar) were significantly lowered in the presence of dantrolene (DANT, 50 µM, blue bar) or levetiracetam (LEV, 100 µM, red bar (*$p<0.05$, compared to GWI, one-way ANOVA, post-hoc Tukey test, n= 5-6 animals for each treatment). Data represented as mean ± SEM.

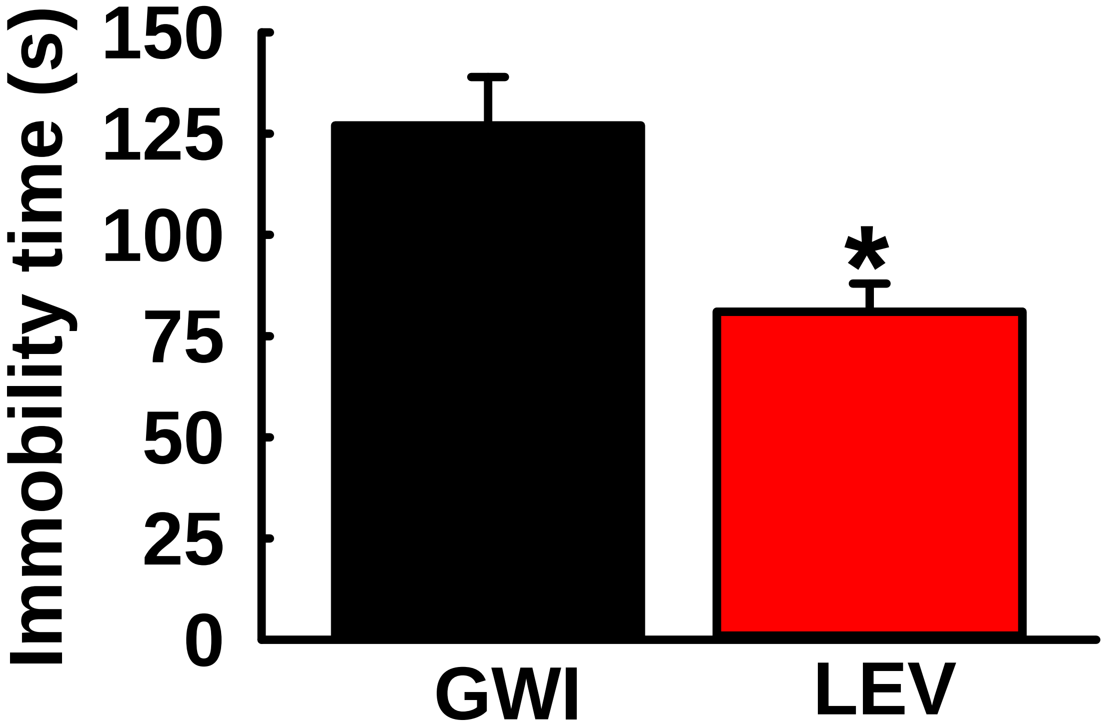

Figure 3A. Antidepressant Effects of Levetiracetam. The immobility time in GWI rats was significantly lowered in the presence of levetiracetam (LEV, 50 mg/kg, i.p.) indicative of a robust antidepressant-like effect. Data expressed as mean ± SEM, *$p<0.05$, t-test, n= 6 rats.

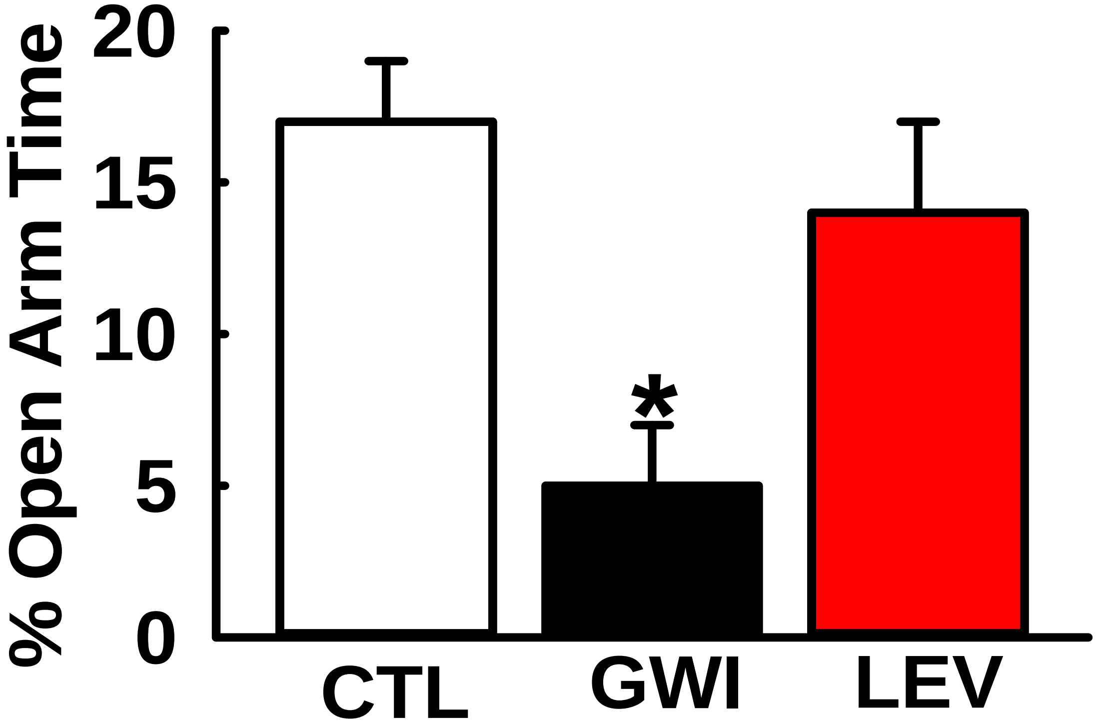

Figure 3B. Anxiolytic Effects of Levetiracetam. GWI rats when tested in the EPM task displayed significantly lower open arm time compared to age-matched control rats indicative of increased anxiety. In the presence of levetiracetam (LEV, 50 mg/kg, i.p.), GWI rats showed a significantly increased exploration of the open-arm suggesting an anxiolytic effect. Data expressed as mean ± SEM, *$p<0.05$, t-test, n= 6 rats.

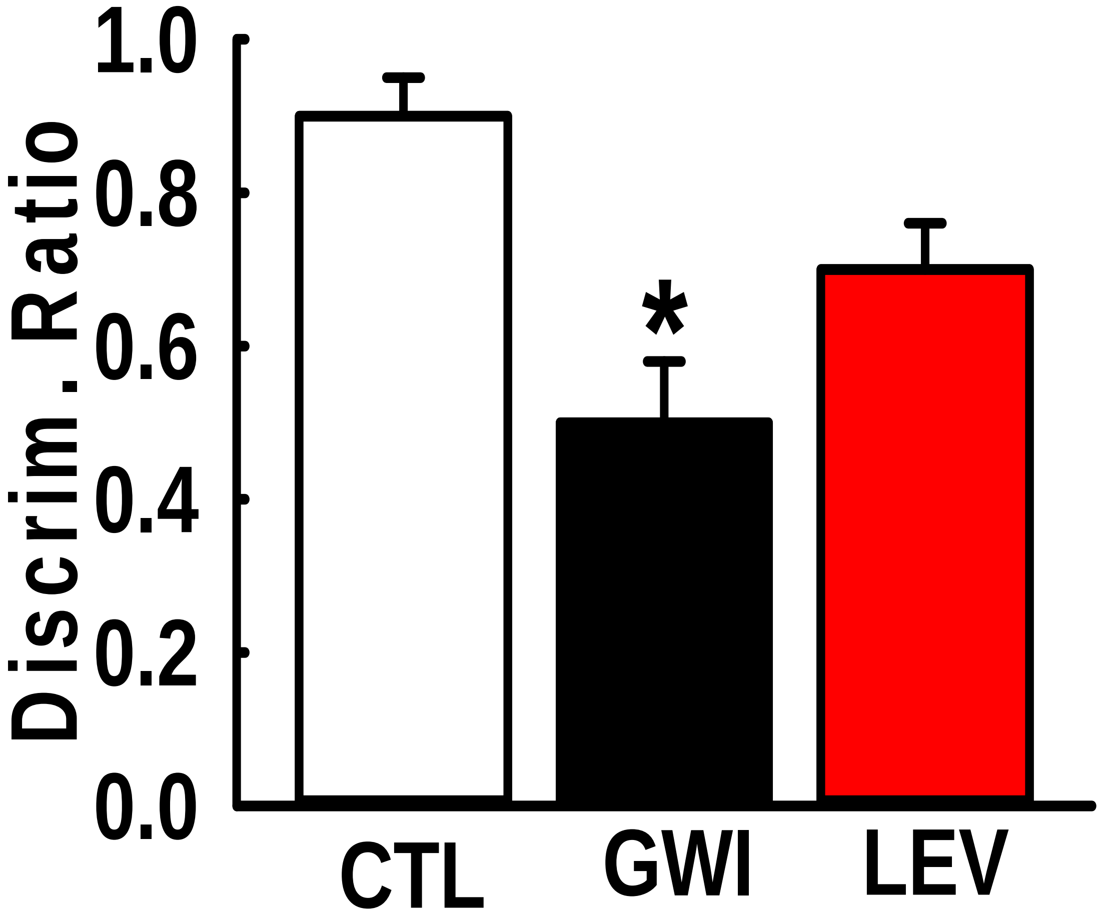

Figure 3C. Effect of Levetiracetam on Memory. GWI rats exhibited a significantly lower discrimination ratio on the NOR test indicative of impaired recognition memory compared to a higher discrimination ratio observed in age matched control rats. In the presence of levetiracetam (LEV, 50 mg/kg, i.p.), there was a significant improvement in the memory performance as indicated by discrimination ratios that were significantly higher than GWI rats. Data expressed as mean ± SEM, *$p<0.05$, t-test, n= 6 rats.

3.3. What opportunities for training and professional development has the project provided?

The GWIRP grant has allowed me to engage high school and undergraduate students in research and spark an interest in GWI research. My laboratory actively participates in VCU Summer Research Program. By informing the young students of GW history, sacrifices made by our Veterans, and how they can make a difference in the lives of GWI suffering Veterans by participating in our research program, my laboratory makes an attempt to get new generation of scientists interested in GWI research. The following students have conducted GWI-related research in 2017:

Name	Affiliation
Ms. Edna Santos	VCU Biology
Ms. Elizabeth Vu	VCU HPEX
Ms. Wasamah Sheikh	VCU Anthropology
Ms. Elizabeth Church	VCU HPEX
Ms. Kathryn Hobbs	UNC Biology
Mr. Richard Wang	TJHSST
Dr. Kristin Phillips	VCU Neurology

3.4 How were the results disseminated to communities of interest?

We presented our work on GWI model development and calcium level estimations to scientists and consumers at the 2017 Society of Toxicology Annual Meeting. All the peer-reviewed manuscripts produced from our GWIRP efforts are freely available via VCU Scholars Compass and PMC Medline. We also actively promote our findings on social media such as Twitter, LinkedIn, and also on open-access scientific platforms.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

"Nothing to report"

4. Impact

This research has offered new molecular targets for drug development and has identified levetriacetam for the effective treatment of GWI neurological symptoms of depression, anxiety, and cognitive deficits.

4.1 What was the impact on the development of the principal discipline(s) of the project?

Our research will have a major impact on the lives of veterans suffering from GWI by providing investigators a novel model of GWI symptoms to identify molecular bases of GWI in search of effective therapeutic options.

4.2 What was the impact on other disciplines?
Exposure to OP agents that is occupational, accidental, or terrorism-related is a legitimate concern. Our work involving model development has the capability to also serve as a rodent model of chronic OP exposure in the civilian population.

4.3 What was the impact on technology transfer?
"Nothing to report"

4.4 What was the impact on society beyond science and technology?
"Nothing to report"

5. Changes/ Problems:
"Nothing to report"

6. Products
6.1 Publications, conference papers, and presentations

6.1.1 Journal publications:
1. Phillips K and Deshpande LS (2017) Chronic Neurological Morbidities and Elevated Hippocampal Calcium Levels in a DFP-Based Rat Model of Gulf War Illness. J Mil Med. *(in press).*

6.1.2 Other publications, conference papers, and presentations
1. K. Phillips, R. Blair, L. Deshpande. Novel Molecular Mechanisms for Neurological Morbidities in a DFP Based Rat Model of Gulf War Illness. In: *The Toxicologist*: Supplement to *Toxicological Sciences*, [Volume-156 (Issue-1)], Society of Toxicology, [2017]. Abstract no. [2885]. [Baltimore, MA, Mar. 12-16, 2017]

7. Participants & Other Collaborating Organizations

7.1 What individuals have worked on the project?

Name	Laxmikant Deshpande	Kristin Phillips	Robert Blair
Project Role	PI	Post-Doc Fellow	Investigator
Research Identifier **eRA commons:**	orcid.org/0000-0003-1491-1561 DESHPANDELS	 KPHILLIPS5	 REBLAIR
Nearest person month worked	6	10	2
Contribution to the project	DFP exposures, behavioral assays, estimation of calcium, data analysis and communication	DFP exposures, preparation of hippocampal slices, estimation of intracellular calcium	Protein isolation and estimations using western blotting
Funding support	DOD, NINDS	DOD	DOD, NINDS

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to report"

7.3 What other organizations were involved as partners?

"Nothing to report"

8. Special reporting requirements

"Not applicable"

9. Appendices

9.1 Bibliography

9.2 SOT Poster

9.1 Bibliography

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Novel Molecular Mechanisms for Neurological Morbidities in a DFP Based Rat Model of Gulf War Illness

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INTRODUCTION

Chronic exposures to organophosphates (OP) including pesticides and nerve gas during the Persian Gulf War has been attributed to the development of a debilitating disorder known as Gulf War Illness (GWI)[1,2]. Despite current treatment recommendations, GWI Veterans continue to suffer from neurological morbidities of depression, and memory impairments amongst others[3].

We have developed an OP-based rat model of GWI that mimics these neurological symptoms in the absence of other confounding factors present in the war theatre[4]. This model is being used to identify molecular correlates for GWI to develop effective treatment solutions. A molecular mechanism that is commonly altered in neurological disorders is aberrant calcium signaling.

Calcium ions (Ca^{2+}) are signaling molecules modulating memory, mood, and behavior functions. Disruptions in neuronal Ca^{2+} are implicated in Alzheimer's, Epilepsy, TBI, conditions which manifest similar neurological morbidities as seen in GWI[5]. However, the status of Ca^{2+} homeostasis in the development of behavioral impairments in GWI is unknown, and thus is the focus of this investigation.

METHODS

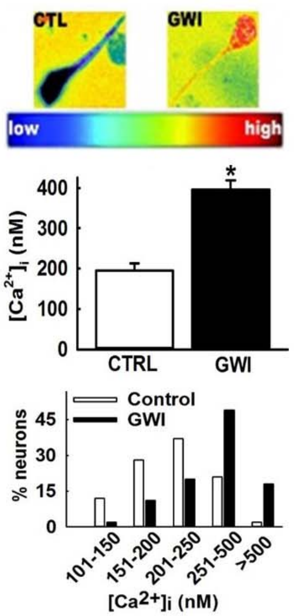
GWI Model: Male rats (S.D., 9-weeks) were injected once daily with DFP (0.5 mg/kg, s.c., ice-cold PBS) for 5-days, while control rats received saline injections[5]. At 3-months post-DFP exposure, rats were tested for the symptoms of depression and cognitive deficits using a battery of behavioral assays as described earlier[4].

Calcium Imaging: CA1 neurons were acutely isolated from hippocampal slices and loaded with fluorescent Ca^{2+} indicator Fura-2AM. They were then stimulated using alternating 340/380 wavelengths and resulting emissions were acquired to record Ca^{2+} transients as described earlier[5].

Calcium Calibration: Calibration curve was constructed using solutions of calibrated Ca^{2+} buffers ranging from Ca^{2+} free to 39 µM Ca^{2+}. Values from the calibration curve were used to convert fluorescent ratios to $[Ca^{2+}]_i$. Final $[Ca^{2+}]_i$ were calculated from the background corrected 340/380 ratios using the Grynkiewicz eq^n: $[Ca^{2+}]_i = (K_d \times Sf_2 / Sb_2) \times (R - R_{min}) / (R_{max} - R)$

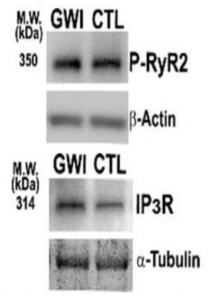
Western Blotting: Hippocampal tissue from GWI rats processed using standard procedures. From each rat, hippocampal homogenates were prepared. Quantitation of the amount of RyR-p, IP3-R, and PLCγ per mg protein in each sample was performed. Antibody specificity was established using blocking peptides and no antibody controls. Internal and external standards to control for loading and sample variability were used.

Elevated hippocampal $[Ca^{2+}]_i$ in GWI neurons



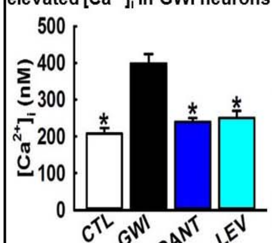
Acutely isolated CA1 neurons from GWI rats manifested $[Ca^{2+}]_i$ of 399 ± 26 nM, that were higher than $[Ca^{2+}]_i$ in neurons from age-matched controls (208 ± 16 nM, n= 7 animals, $p<0.05$, t-test). Analysis of the population distributions of $[Ca^{2+}]_i$ revealed a right-ward shift towards higher Ca^{2+} concentrations in GWI neurons compared to control neurons ($p<0.01$, ψ^{-2} test, n= 161 neurons).

Increased CICR protein expression in GWI rats



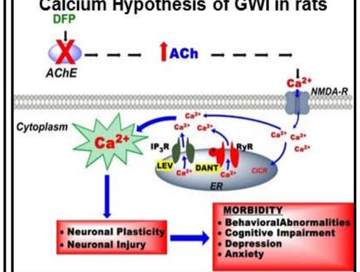
Western blot analysis showed increased levels of intracellular Ca^{2+} release receptor proteins P-RyR_2 (100 ± 14.2%, 124.2 ± 31.9%) and IP_3-R (100.0 ± 48.6%, 105.8 ± 43.2%).

CICR antagonists lower elevated $[Ca^{2+}]_i$ in GWI neurons



Dantrolene (DANT, 50 µM) or levetiracetam (LEV, 100 µg) lowered elevated $[Ca^{2+}]_i$ in GWI neurons (240 ± 11 nM and 250 ± 19 nM respectively, n= 5 animals, $p<0.05$, t-test).

Calcium Hypothesis of GWI in rats



CONCLUSIONS

We observed that GWI rats manifested chronic elevations in hippocampal Ca^{2+} levels. The protracted Ca^{2+} elevation had its origin in Ca^{2+} release from intracellular Ca^{2+} stores, since the ryanodine/IP_3 receptor antagonists produced a significant reduction in elevated $[Ca^{2+}]_i$ in GWI neurons. These sustained $[Ca^{2+}]_i$ elevations appear to be due an increased expression in major components of intracellular Ca^{2+} release machinery, particularly the RyR_2 receptor. Mechanisms underlying increased expression of P-RyR_2 are being investigated.

Since Ca^{2+} is a major second messenger molecule, such chronic increases in its levels could activate signaling cascades that could underlie the neuronal damage and also produce pathological synaptic plasticity that expresses itself as GWI morbidity. Treatment with drugs targeted at blocking this enhanced intracellular Ca^{2+} release could be effective therapies for GWI related neurological morbidities.

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